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14. ABSTRACT

This document provides an annual progress report for the grant W81XWH-13-1-0485 entitled "Ovarian Cancer Biomarkers That Predict Response to PARP Inhibitors and Platinum." Over the past 12 months we have i) completed validation of the immunohistochemical assays for components of the NHEJ pathway, ii) successfully transferred these assays to the Mayo Clinic Pathology Research Core laboratory, which optimized them for performance on an autostainer; and iii) continued acquisition of tissue specimens from the initially proposed 180-patient phase 2 rucaparib trial (ARIEL2, part 1– ClinicalTrials.gov identifier NCT01891344), which will be subjected to IHC staining and scoring as one batch per the deliberations of the statisticians associated with this grant and the ARIEL2 trial. A more complete abstract is provided on page 4 of this document.

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Table of Contents

ABSTRACT

The breast and ovarian cancer susceptibility genes *BRCA1* and *BRCA2* (*BRCA1/2*) are key components of the Fanconi anemia (FA)/homologous recombination (HR) pathway of DNA repair. Previous work had shown that cancer cells with deleterious FA/HR pathway mutations are hypersensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. Importantly, however, only about half of the cancer patients with germline FA/HR pathway mutations respond to PARP inhibitors, raising the question of why a substantial fraction of HR-deficient cancers are resistant to these agents in the clinic. Based on previous work in the Swisher and Kaufmann laboratories, we proposed to test the **hypothesis** that *two different conditions must be met for ovarian cancer to be hypersensitive to platinum and PARP inhibitors: The FA/HR pathway must remain disabled and NHEJ must remain intact and functional.* Although we proposed two aims, the aim in previously banked specimens was removed before the present grant was awarded, leaving us with the following aim: **Correlate biomarkers of HR deficiency and NHEJ pathway integrity in pre-treatment biopsies with response to a PARPi in a prospective single-agent PARPi phase 2 clinical trial in sporadic ovarian carcinoma.** Over the past 12 months we have i) completed validation of the immunohistochemical assays for components of the NHEJ pathway, ii) successfully transferred these assays to the Mayo Clinic Pathology Research Core laboratory, which optimized them for performance on an autostainer; and iii) continued acquisition of tissue specimens from the initially proposed 180-patient phase 2 rucaparib trial (ARIEL2, part 1–ClinicalTrials.gov identifier NCT01891344), which will be subjected to IHC staining and scoring as one batch per the deliberations of the statisticians associated with this grant and the ARIEL2 trial.

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme that regulates five different DNA repair pathways. Building on preclinical observations that defects in homologous recombination (HR) repair, which are found in 30-50% of ovarian cancers, sensitize cells to killing by PARP inhibitor, five separate phase 3 trials involving PARP inhibitors have opened in ovarian cancer. During the past year, the Food and Drug Administration approved the PARP inhibitor olaparib for women with recurrent ovarian cancer and inherited mutations in the *BRCA1* and *BRCA2* genes. During the same period, the PARP inhibitor rucaparib was granted breakthrough status by the FDA. In collaboration with Elizabeth Swisher (University of Washington), the present synergistic translational leverage project is assessing multiple aspects of DNA repair pathway integrity in pretreatment biopsies from a large multi-institution phase 2 study of rucaparib that focused on enrolling predominantly women without inherited mutations in the *BRCA1* and *BRCA2* genes in order to develop a biomarker of PARP inhibitor responsiveness for non-BRCA carriers (the 180-patient initial cohort of the Clovis Oncology-sponsored ARIEL2 biomarker clinical trial). In particular, the Kaufmann laboratory is using immunohistochemistry to assess expression of proteins in the nonhomologous end-joining (NHEJ) pathway (53BP1, Ku70, Ku80, DNA-PKcs, XRCC4, DNA ligase IV) as well as PARP1. This group of proteins was chosen based on our preclinical studies showing that PARP inhibitors activate the error-prone NHEJ repair pathway in homologous recombination-deficient ovarian cancer cells (1, 2) and that loss of any of the proteins in this pathway will simultaneously impair NHEJ and PARP inhibitor-induced killing (*op. cit.* and additional unpublished observations).

Key words: ovarian cancer, drug resistance, rucaparib, phase 2, DNA repair, homologous recombination, nonhomologous end-joining (NHEJ), immunohistochemistry poly(ADP-ribose) polymerase (PARP1), BRCA1, BRCA2, Ku70, Ku80, 53BP1, DNA-PK, DNA ligase IV, XRCC4.

Overall Project Summary:

Based on recommendations following submission of last year's progress report, the following progress is broken down by tasks in our statement of work. Dr. Swisher will provide progress on tasks that she is carrying out in her report. To date, we have achieved all projected milestones on time.

Specific Aim 1 (specified in revised technical abstract)	Timeline	Site 2	Status
Subtask 1: submission of Mayo IRB approvals and related material for DOD's HRPO approval/exempt finding and MTA with Clovis Oncology.	1	Dr. Kaufmann	Completed
Subtask 2: Collect FFPE sections of 200 pre-treatment tumor biopsies coordinated through Clovis and evaluate with IHC of NHEJ proteins	2-33	Dr. Kaufmann	All biopsies collected by Clovis. Transfer to Kaufmann laboratory in progress (see text).
Subtask 3: Collect FFPE sections from 200 paired primary carcinoma blocks from time of diagnosis for all enrolled patients, coordinated through Clovis evaluate with IHC of NHEJ proteins.	6-33	Dr. Kaufmann	All biopsies collected by Clovis. Transfer to Kaufmann laboratory in progress (see main text).

Subtask 1: Consistent with our Statement of Work, we prepared paperwork for the IRB and HRPO regarding the analysis of deidentified samples from the phase 2 rucaparib trial. Both determined that the research was exempt.

Subtask 2: As described in the 2014 progress report but not explicitly stated, we have broken this subtask into four activities:

- Validation of staining
- Transfer into the CLIA-like environment of the Mayo Clinic Pathology Research Core
- Trial sample acquisition
- Staining of trial samples for NHEJ proteins and PARP1

This progress report provides an update on these four activities.

Validation of staining/transfer into the CLIA-like environment of the Mayo Clinic Pathology Research Core: At the time of the 2014 progress report, we provided evidence of successful and selective staining for DNA-PKcs, Ku70 and XRCC4. In each case staining was observed in formalin fixed/paraffin embedded cell lines that express the protein of interest but not in cell lines that lack the protein of interest due to siRNA-mediated knockdown or gene interruption. Each of these assays has been successfully transferred to the Mayo Clinic Pathology Research Core, where conditions have been optimized on an autostainer to allow greater batch-to-batch reproducibility. The antibodies used and assay outputs are identical to those reported in that 2014 progress report.

At the time of the 2014 progress report, a number of the other assays were problematic. Those assays have now been successfully optimized as described here and also transferred to the Pathology Research Core.

53BP1: At the time of the 2014 progress report, the 53BP1 staining was plagued by high cytoplasmic background. After examining two separate 53BP1 rabbit polyclonal antibodies that gave good results on immunoblots (similar to panel A in Figure 1) but had high nonspecific binding on formalin fixed, paraffin embedded (FFPE) cells, we settled on a mouse monoclonal antibody suggested by Dr. Swisher that yielded differential staining in FFPE samples (Figure 1). Under the conditions of this assay, we see a range of 53BP1 expression intensities in FFPE clinical ovarian cancer specimens (not shown). This assay has been transferred to the Pathology Research Core, which stained the samples shown in Figure 1.

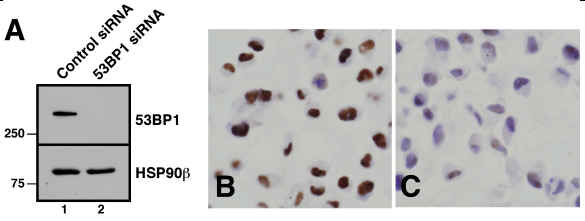

Figure 1. Validation of 53BP1 staining. A, immunoblot for 53BP1 in Ovcar8 ovarian cancer cells transfected with control siRNA (lane 1) or 53BP1 siRNA (lane 2). Heat shock protein 90β (HSP90β) served as a loading control. Parallel aliquots of these same cells were fixed in formalin and embedded in paraffin to provide material for the optimization of staining shown in panels B and C. **B, C,** FFPE cells transfected with control siRNA (B) or 53BP1 siRNA (C) were stained with anti-53BP1 as described in the main text. Note marked decrease in IHC signal with 53BP1 knockdown.

Ku80: At the time of the 2014 progress report the Ku80 staining showed nice nuclear localization and variability in intensity among different ovarian cancer clinical specimens. However, after Ku80 knockdown, the signal in FFPE cell lines was unaltered. With the realization that the previously used antibody lacked specificity, we studied two additional anti-Ku80 antibodies and demonstrated that Abcam ab79391 shows staining that is nuclear (as Ku80 is known to be) and varies appropriately depending on the antigen expression (Figure 2). This assay has been transferred to the Pathology Research Core, which stained the samples shown in Figure 2.

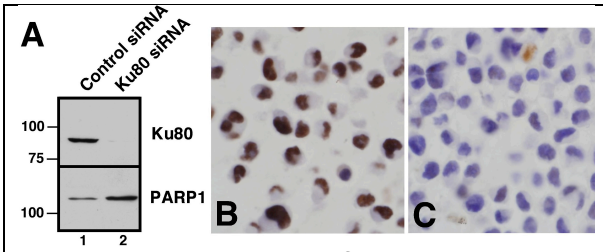

Figure 2. Validation of Ku80 staining. A, immunoblot for Ku80 in Ovcar8 cells transfected with control siRNA (lane 1) or Ku80 siRNA #5 (lane 2). PARP1 served as a loading control. **B, C,** FFPE cells transfected with control siRNA (B) or Ku80 siRNA #5 (C) were stained with anti-Ku80 as described in the main text.

PARP1: At the time of the 2014 progress report the PARP1 staining showed nice nuclear localization and variability in intensity among different ovarian cancer clinical specimens. However, the supposed *PARP1*$^{-/-}$ lines obtained for validation also showed staining. Accordingly, we obtained additional *PARP1*$^{-/-}$ cells, demonstrated lack of PARP1 protein expression (shown in Figure 3A for clone 22-5), and identified a PARP1 antibody that stains nuclei from PARP1-containing cells but not PARP1$^{-/-}$ cells. This assay has been transferred to the Pathology Research

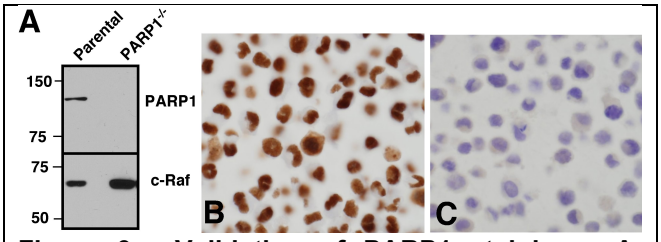

Figure 3. Validation of PARP1 staining. A, immunoblot for PARP1 in parental and *PARP1* gene targeted HCT116 cells. c-Raf served as a loading control. **B, C,** FFPE parental HCT116 cells (B) or *PARP1*$^{-/-}$ HCT116 cells (C) were stained with anti-PARP1 as described in the main text

Core, which stained the samples shown in Figure 3. Additional staining of their inhouse ovarian cancer tissue microarray showed variable staining among ovarian cancers similar to that reported in the 2014 progress report.

DNA ligase IV: At the time of the 2014 progress report, no staining was observed in any ovarian cancer specimen with the antibody being utilized. We subsequently tested a Santa Cruz Biotechnology antibody that was credentialed for IHC but observed nuclear staining that lacked specificity, i.e., was unaltered in cells that contained diminished DNA ligase IV by immunoblotting after siRNA-mediated knockdown. Our subsequent experiments have shown that Abcam ab193353, a rabbit monoclonal antibody developed by Epitomics, exhibits the expected nuclear staining in Ovcar8 cells that is diminished by DNA ligase IV siRNA (Figure 4). Based on these recent results, we are in the process of transferring this assay to the Pathology Research Core for further optimization on an autostainer.

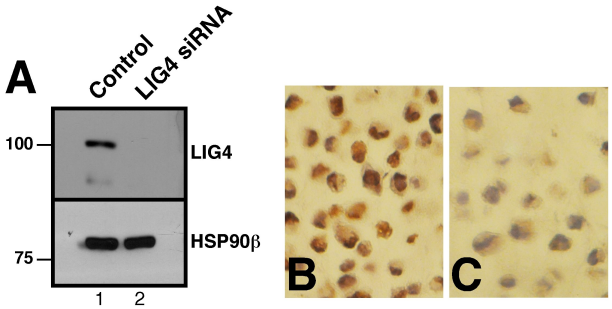

Figure 4. Initial identification of specific antibody for DNA ligase IV staining. A, immunoblot for DNA ligase IV (LIG4) in Ovcar8 cells transfected with control siRNA (lane 1) or DNA ligase IV siRNA (lane 2). Heat shock protein 90β (HSP90β) served as a loading control. **B, C,** FFPE cells transfected with control siRNA (B) or DNA liagase IV (C) were stained with anti-DNA ligase IV as described in the main text. Titration of the antibody and transfer to the Pathology Research Core continues.

Artemis: We have tested (and continue to test) a series of anti-Artemis antibodies. In our hands Artemis tagged with a variety of epitope tags (N-terminal FLAG tag, C-terminal S peptide tag) localizes to the nucleus after transfection. In contrast, many of the commercial anti-Artemis antibodies show extensive cytoplasmic staining. Others, unfortunately, show nuclear staining that does not diminish when Artemis is knocked down. Based on our previous experience in raising a large number of antibodies to other proteins over the years (3-9), we also used Institutional funds to try to raise a murine monoclonal antibody but did not have success. We will continue to test antibodies (most recently Abcam ab151512) but are concerned that this is not an easy antigen to raise antibodies with suitable specificity.

Trial sample acquisition/Staining of trial samples for NHEJ proteins and PARP1: The phase 2 clinical trial that is providing samples for the correlative assays in the Kaufmann and Swisher laboratories (ClinicalTrials.gov identifier NCT01891344) completed enrollment in November of 2014. Preliminary data on response rates were so promising that rucaparib was assigned breakthrough designation by the FDA and Clovis decided to amend ARIEL2 to enroll a second cohort of patients to provide additional data for FDA approval.

To date the Kaufmann laboratory has received slides from 25 pretreatment biopsies. As of last week Foundation Medicine has completed sequencing of *BRCA1* and *BRCA2* in the remaining pretreatment biopsies. Accordingly, Kevin Lin (Principal Scientist, Cancer Genomics at Clovis) has indicated that the remaining samples will be sectioned and shipped to the Kaufmann laboratory within the next several weeks. Dr. Lin also confirmed that slides from surgical specimens obtained at the time of initial diagnosis will also be provided, as they have been to Dr. Swisher. We anticipate receiving all of these materials by the end of November, 2015. They will be immediately transferred to the Pathology Research Core for staining and to Dr. Visscher for scoring.

Key research accomplishments

Preliminary results from the ARIEL2 study (initially designed by Drs. Swisher and Kaufmann as described in the original grant application) have led to FDA breakthrough designation for rucaparib and were presented at ASCO in a plenary session. The first manuscript of these data is in preparation.

Conclusions

The Kaufmann lab is on track to complete staining and scoring of all ARIEL2 samples by month 30 and begin putting data together with Dr. Swisher and correlating the combined data with clinical outcomes.

Publications

Scott CL, Swisher EM, Kaufmann SH. Poly (ADP-ribose) polymerase inhibitors: recent advances and future development. *J Clin Oncol.* 2015;33(12):1397-406. PMCID: PMC4517072

Abstracts and presentations

None

Inventions, patents and licenses

None

Other achievements

None

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Appendices

None